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Nitric Oxide Attenuates Normal and Sickle Red Blood Cell Adherence to Pulmonary Endothelium

Sharon L. Space,[1,3]* Peter A. Lane,[1,4] Cheryl K. Pickett,[2,3] and John V. Weil[2,3]

[1]Department of Pediatrics, University of Colorado Health Sciences Center, Denver, Colorado
[2]Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado
[3]Cardiovascular Pulmonary Research Laboratory, University of Colorado Health Sciences Center, Denver, Colorado
[4]Colorado Sickle Cell Treatment and Research Center, University of Colorado Health Sciences Center, Denver, Colorado

Increased adherence of sickle red blood cells (RBC) to endothelium is implicated as an initiating event of vaso-occlusion in sickle cell disease. Although much is known about the humoral influences of this interaction, there has been little investigation regarding endothelial contributions. Endothelial derived nitric oxide (NO) inhibits adhesion of platelets and leukocytes to endothelium and decreases expression of VCAM-1, an endothelial adhesion site implicated in sickle RBC/endothelial adherence. However, whether NO inhibits RBC adherence to endothelium is unexplored. We tested this hypothesis with endothelial monolayers exposed to RBC from normal (Hb AA) and sickle cell (Hb SS) volunteers in a parallel plate flow chamber. To decrease NO production, endothelial monolayers were exposed to 100 μM nitro-L-arginine (NLA), an inhibitor of nitric oxide synthase, resulting in an 87% increase in normal RBC adherence ($P = 0.002$). Because adherence of normal RBC to endothelium was low, the effect of DETA-NO, an NO donor, was tested after activation of endothelium with TNF-α increased adherence by 130% ($P < 0.001$). Subsequent addition of 2 mM DETA-NO produced a 75% decrease in adherence of normal RBC to endothelium ($P = 0.03$). At baseline, sickle RBC were significantly more adherent than normal RBC ($P < 0.001$) and DETA-NO decreased sickle RBC adherence by 54% ($P = 0.04$). Thus, NO inhibits both normal and sickle RBC adherence to endothelium. Strategies that enhance NO activity may be therapeutic in sickle cell disease. Am. J. Hematol. 63:200–204, 2000. © Wiley-Liss, Inc.

Key words: sickle cell anemia; nitric oxide; cell adhesion

INTRODUCTION

The exaggerated adherence of sickle RBC to endothelium has been implicated as an early step in vaso-occlusion, a hallmark of sickle cell disease [1–3]. Numerous studies have investigated the role of humoral and RBC-related modulators of sickle RBC adherence to endothelium [4–10]. However, little is known about the role of endothelial derived factors, such as nitric oxide (NO), in this interaction

Several lines of evidence support the hypothesis that NO might decrease RBC/endothelial adherence. Although NO inhibits adherence of white blood cells and platelets to endothelium, its direct effects on RBC adherence to endothelium have not been previously reported. Several in vitro studies provide indirect support for the hypothesis that NO attenuates RBC adherence to endothelium. NO donors decrease endothelial expression of VCAM-1 [11], an endothelial receptor involved in RBC adherence [4,6,9]. Further, in vivo studies in rats infused with sickle RBC showed that inhibition of nitric oxide synthase (NOS) increased cerebral RBC retention and the incidence of stroke and death. It is unclear if this is due to a vasomotor or an adherence effect [12]. We have found no direct studies of the effect of NO on RBC/endothelial adherence.

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*Correspondence to: Sharon L. Space, M.D., CVP Research, Campus Box B133, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262. E-mail: sharon.space@uchsc.edu

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Accordingly, we sought to test the idea that NO inhibits endothelial adherence of sickle and normal RBC. To this end, we investigated the effect of NO inhibitors and donors on in vitro adherence of RBC to cultured endothelium under flow conditions. Our findings suggest that NO is an inhibitor of RBC/endothelial adherence.

METHODS

Subjects

After obtaining informed consent under an IRB-approved protocol, blood was collected by venipuncture in sodium heparin from normal volunteers (Hb AA) and patients with sickle cell anemia (Hb SS). Patients aged 8–15 years had no symptoms of vaso-occlusion and had not received PRBC transfusions for at least 4 months.

RBC Suspensions

Blood was centrifuged at 2,100 rpm for 15 min within 8 hr of collection, and the plasma and buffy coat were removed. RBC were washed twice with saline and resuspended at a hematocrit of 1% in Hepes (10 mM, Sigma Chemical Co., St. Louis, MO)–Hanks Balanced Salt Solution (HBSS, 0.185 g/L calcium chloride·$2H_2O$, 0.09767 g/L anhydrous magnesium sulfate, 0.4 g/L potassium chloride, 0.06 g/L anhydrous potassium phosphate monobasic, 8.0 g/L sodium chloride, 0.04788 g/L anhydrous sodium phosphate dibasic, 1.0 g/L D-glucose, 0.35 g/L sodium bicarbonate; Sigma Chemical Co., St. Louis, MO) with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO).

Endothelial Cell Cultures

Endothelial cells were isolated from bovine pulmonary arteries as described previously [13]. Primary cultures were grown to confluence (approximately 7 days) and subcultured with trypsin every 7–10 days. Subcultures from passages two through six were used for the experiments. Cells were inspected by phase contrast microscopy and identified as endothelial cells by the typical cobblestone morphology.

For use in the parallel plate flow chamber, cultured endothelial cells were suspended with trypsin and diluted with 20% fetal calf serum in D-valine, nonessential amino acids, penicillin, and streptomycin and seeded on glass slides coated with 1% gelatin. They were fed at 4–12 hr and grown to confluence over 3–4 days.

Adhesion Assay

Slides bearing confluent endothelial monolayers were placed in the parallel plate flow chamber, similar to that described by Barabino in 1987 [3] and maintained at a temperature of 37°C with a servo-controlled heating unit attached to the chamber. RBC suspensions and media were also maintained at 37°C in a water bath (Precision Scientific, Model 183). The flow rate via a syringe pump (Harvard Apparatus, Model PHD 2000) was 0.191 mL/min to achieve a sheer stress of 1 dyne/cm^2. Preparations were observed with an inverted microscope at 20× (Olympus, Model CK2), connected to a video camera (Javelin, Model JE-3662RGB), monitor (SONY, Model PVM-1354Q), and VCR (Panasonic, Model PV-S4690).

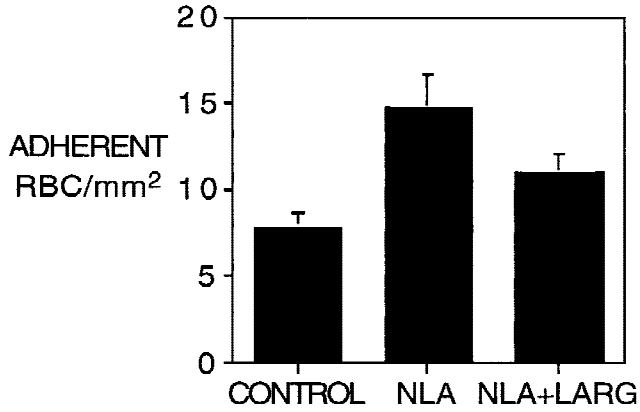
Experimental Protocols

Experiments began with a 30–60 min pretreatment period during which endothelial monolayers were incubated with 20% fetal calf serum/D-valine (control conditions), with or without cytokines, NO inhibitors, and/or NO donors. Following pretreatment, endothelial covered slides were placed in the flow chamber. Monolayers were then perfused with a 5 min pre-rinse with Hepes–HBSS–BSA (pH 7.3–7.4), 10 min of RBC perfusion, 5 min of stopped flow (static incubation), and a 20 min perfusion with Hepes–HBSS–BSA to remove non-adherent RBC. Adherent RBC were counted in a minimum of 24 random fields and reported as the number of adherent RBC/mm^2.

Control. In control runs endothelium was pretreated with 20% fetal calf serum/D-valine.

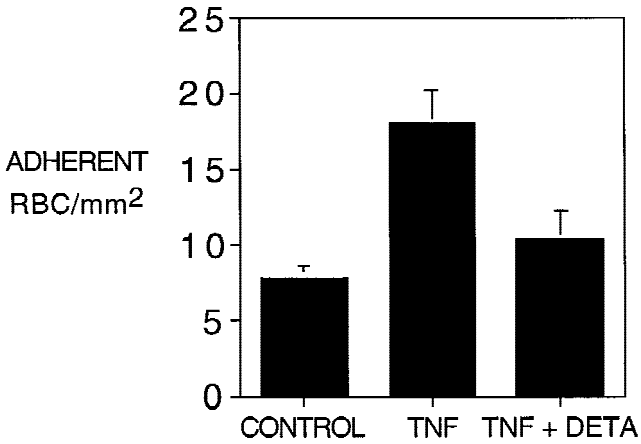
NO inhibition. Endothelial monolayers were pretreated with nitro-L-arginine (NLA, 1 μM or 100 μM, Sigma Chemical Co., St. Louis, MO), an inhibitor of nitric oxide synthase (NOS), diluted in 10 mL 20% fetal calf serum/D-valine for 30 min. To control for nonspecific effects of NLA, endothelial monolayers were pretreated with L-arginine (100 μM) and NLA for 30 min. As an added control, additional endothelial monolayers were treated with L-arginine (100 μM) alone. The same concentration of NLA, L-arginine, or both was added to the RBC suspension and media just prior to the start of the experiment so that endothelial monolayers and RBC were exposed to the intervention throughout the experiment.

NO donor. Because normal RBC/endothelial adherence was low, the effect of NO donors was tested after adherence of normal RBC was enhanced by cytokine activation of endothelium [4,5]. Initial dose–response experiments were run to determine the minimal dose and duration of TNF-α exposure that produced a consistent measurable increase in RBC adherence to endothelium with minimal damage to endothelial monolayers. Doses ranged from 0.5 to 500 units/mL, with exposure varying from 30 min to 6 hr. The minimal dose and duration which produced a consistent, measurable increase in adherence was 5 units/mL for 1 hr. Increasing the dose and duration of exposure resulted in further increases in RBC adherence but was associated with significant changes in shape with retraction and gap formation of monolayer cells. Thus, for all reported experiments we used the minimal dose which produced a consistent elevation in adherence with minimal damage to the endothelial monolayer. Endothelial monolayers were pretreated with



MEAN	7.8	14.6	10.9
SEM	0.82	2.08	1.12
N	19	19	7

Fig. 1. Inhibition of nitric oxide increases normal RBC/endothelial adherence. Decreasing NO by exposure of endothlial monolayers and RBC to nitro-L-arginine (NLA), an inhibitor of NO generation, significantly increases adherence of normal RBC to endothelium ($P = 0.002$). This effect is partially blocked by addition of L-arginine, the substrate for NO generation (P = NS).



MEAN	7.8	17.9	10.3
SEM	0.82	2.33	1.84
N	19	9	5

Fig. 2. Increased nitric oxide blocks cytokine-activated endothelial adherence of normal RBC. Activation of endothelium with the cytokine TNF-α doubles adherence of normal RBC to endothelium ($P < 0.001$). Addition of the NO donor, DETA-NO, blocks this cytokine-induced increase in RBC/endothelial adherence ($P = 0.03$).

a dose of 5 units/mL TNF-α (mouse recombinant, expressed in *Escherichia coli,* Sigma Chemical Co., St. Louis, MO) for 1 hr, with or without the addition of DETA-NONOate [NOC-18,DETA/NO, (*Z*)-1-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium 1,2-diolate; (DETA-NO, 2,000 μM, Alexis Corporation, San Diego, CA), an NO donor, or vehicle control after 30 min, prior to placement in the flow chamber. Since sickle RBC were much more adherent to endothelium, no TNF-α activation was used before exposure to the NO donor. Endothelial monolayers were pretreated with 2,000 μM DETA-NO alone for 30 min prior to placement in the flow chamber and perfusion with sickle RBC. To maintain exposure to the NO donor throughout the experiments, DETA-NO (2,000 μM) was added to normal and sickle RBC suspensions and media just prior to placing endothelial monolayers in the flow chamber.

RESULTS

Normal RBC exhibited a small, but measurable adherence to endothelium which was enhanced by exposure of endothelium and RBC to NLA, an inhibitor of NO generation (Fig. 1). NLA at concentrations of 1 and 100 μM produced an 87% increase in adherence. The addition of L-arginine partially blocked this increase. Exposure to L-arginine alone did not affect baseline adherence for normal RBC (data not shown).

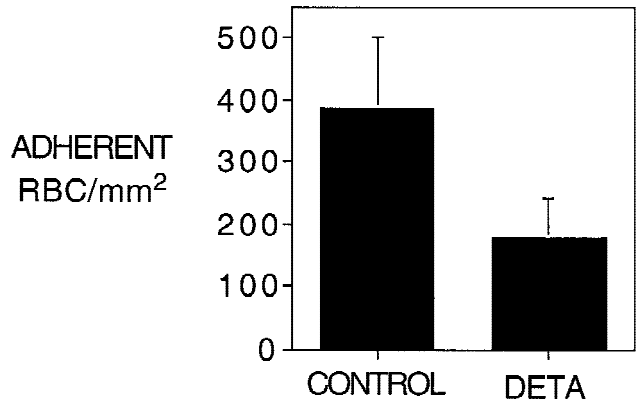
Because adherence of normal RBC was very low, we tested the effect of NO donors after cytokine activation of endothelial monolayers. Exposure to the cytokine TNF-α increased adherence by 120% (Fig. 2). Addition of the NO donor DETA-NO produced a 75% decrement in the cytokine-induced enhancement of normal RBC adherence.

Sickle RBC were much more adherent than normal RBC (387.4 ± 110.81 vs. 7.8 ± 0.82, $P < 0.001$). Thus monolayers used in experiments with sickle RBC were not activated with TNF-α prior to treatment with the NO donor. Exposure of endothelium and RBC to DETA-NO, an NO donor, resulted in a 54% decrease in sickle RBC/endothelial adherence (Fig. 3).

DISCUSSION

We found that the endothelial adherence of normal RBC was enhanced by inhibitors of NO production and that increased adherence of normal RBC, induced by TNF-α, was reduced by an NO donor. Consistent with previous reports [1,3], we found that sickle RBC were significantly more adherent to endothelium than normal RBCs and that this enhanced adherence was significantly diminished by an NO donor. Collectively these findings suggest that endogenous and exogenous NO inhibits endothelial adherence of normal and sickle RBC.

In the present study we used bovine pulmonary artery endothelial cells which have the advantage of ready



MEAN	387.4	177.2
SEM	110.81	63.79
N	6	6

Fig. 3. Nitric oxide decreases sickle RBC/endothelial adherence. Exposure to the NO donor, DETA-NO, significantly decreases endothelial adherence of sickle RBC ($P = 0.04$).

availability and study at low passage. Because the lung is a target organ in sickle cell disease, we chose to investigate adherence of RBC to pulmonary endothelium [14,15]. Although there is concern regarding cross-species effects in the interaction of human RBC with bovine endothelium, our findings reproduce the enhanced adherence of sickle vs. normal RBC as well as the enhancement of normal RBC adherence after exposure to TNF-α previously reported with "all-human" models [1,2,4,5], and in studies with cross-species model systems [2,12]. These consistencies support our approach as a valid method.

Our work also differs from previous reports with respect to our use of lesser exposure to TNF-α. Prior studies used a minimum of 4 hr of exposure to 500 units/mL of TNF-α [5]. With initial dose/time ranging, we found a consistent, reproducible increase in RBC adherence after a 1 hr exposure to 5 units/mL of TNF-α, with no evidence of endothelial cell shape change. With higher doses and longer duration of exposure, we observed shape changes in the endothelial cells with retraction of cells and gap formation and decreased adherence of RBC. We chose the minimal dose and duration that produced a consistent increase in RBC adherence without affecting the confluence of the endothelial monolayer.

The use of a combination of steady/stop-flow conditions also differs from most prior studies. We used this approach because we believe it mimics the intermittent flow conditions in the pulmonary vasculature. In addition, recent reports describe "circulatory stasis" or sluggish, intermittent flow in the mesenteric vessels of transgenic sickle mice as compared to continuous flow in the vessels of normal animals. Thus, combined steady/stop-flow conditions may provide good simulation of in vivo conditions for the assessment of RBC/endothelial interaction [16]. Use of this approach might explain our finding of higher sickle RBC adherence than previously reported in continuous flow protocols [3,10]. We have found one prior study that used a combination of static and continuous flow conditions to measure adherence of sickle WBC and RBC to endothelium and found increased endothelial adherence of sickle WBC and RBC compared to normal cells in a static adhesion assay and with a 10 min static incubation period preceding flow. Adherence of sickle and normal cells were equal using a continuous flow assay [17].

The present work does not address the mechanism by which NO decreases RBC adherence to endothelium. Both RBC and endothelial cells were exposed to NO inhibitors and donors throughout all experiments, making it is unclear if the effect is on the RBC, or endothelium, or both. It is also unclear whether changes in specific receptors mediate this action.

Several lines of clinical evidence point to the potential importance of NO in sickle cell disease. Patients with sickle cell disease often have elevated levels of NO metabolites compared to normal [18,19]. During vaso-occlusive crisis elevated levels of NO metabolites are associated with lower clinical pain scores, suggesting that enhanced NO activity may decrease the severity of vaso-occlusive crisis [20]. Recent studies suggest additional potential beneficial actions of NO in sickle cell disease. These include NO-induced augmentation of oxygen affinity and decreased sickling of sickle RBC [21,22]. Several studies also report decreased circulating levels of L-arginine, the substrate for NO, in patients with sickle cell disease [18,23], which may reflect increased arginine consumption [23] or increased urinary excretion [24].

Together with our findings of the anti-RBC/endothelial adherence effects of NO, these findings suggest that strategies which enhance NO activity, such as supplemental L-arginine, nitrovasodilators, and inhaled NO, may be useful in the prophylaxis and treatment of the vaso-occlusive complications of sickle cell disease.

REFERENCES

1. Hebbel RP, Boogaerts MA, Eaton JW, Steinberg MH. Erythrocyte adherence to endothelium in sickle cell anemia. A possible determinant of disease severity. N Engl J Med 1980;302(18):992–995.
2. Hebbel RP. Perspectives series: cell adhesion in vascular biology. Adhesive interactions of sickle erythrocytes with endothelium. J Clin Invest 1997;99(11):2561–2564.
3. Barabino GA, McIntire LV, Eskin SG, Sears DA, Udden M. Endothelial cell interactions with sickle cell, sickle trait, mechanically injured, and normal erythrocytes under controlled flow. Blood 1987;70(1):152–157.

4. Swerlick RA, Eckman JR, Kumar A, Jeitler M, Wick TM. α-4-β-1-Integrin expression on sickle reticulocytes: Vascular cell adhesion molecule-1-dependent binding to endothelium. Blood 1993;82(6): 1891–1899.
5. Vordermeier S, Singh S, Biggerstaff J, et al. Red blood cells from patients with sickle cell disease exhibit an increased adherence to cultured endothelium pretreated with tumour necrosis factor (TNF). Br J Haematol 1992;81(4):591–597.
6. Setty BN, Stuart MJ. Vascular cell adhesion molecule-1 is involved in mediating hypoxia-induced sickle red blood cell adherence to endothelium: Potential role in sickle cell disease. Blood 1996;88(6):2311–2320.
7. Natarajan M, Udden MM, McIntire LV. Adhesion of sickle red blood cells and damage to interleukin-1 beta stimulated endothelial cells under flow in vitro. Blood 1996;87(11):4845–4852.
8. Kaul DK, Chen D, Zhan J. Adhesion of sickle cells to vascular endothelium is critically dependent on changes in density and shape of the cells. Blood 1994;83(10):3006–3017.
9. Gee BE, Platt OS. Sickle reticulocytes adhere to VCAM-1. Blood 1995;85(1):268–274.
10. Brittain HA, Eckman JR, Wick TM. Sickle erythrocyte adherence to large vessel and microvascular endothelium under physiologic flow is qualitatively different. J Lab Clin Med 1992;120(4):538–545.
11. Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc Natl Acad Sci USA 1996;93(17):9114–9119.
12. French JA, Kenny D, Scott JP, et al. Mechanisms of stroke in sickle cell disease: Sickle erythrocytes decrease cerebral blood flow in rats after nitric oxide synthase inhibition. Blood 1997;89(12):4591–4599.
13. Stelzner TJ, Weil JV, O'Brien RF. Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. J Cell Physiol 1989;139(1):157–166.
14. Haynes J, Jr, Kirkpatrick MB. The acute chest syndrome of sickle cell disease. Am J Med Sci 1993;305(5):326–330.
15. Weil JV, Castro O, Malik AB, Rodgers G, Bonds DR, Jacobs TP. NHLBI Workshop Summary. Pathogenesis of lung disease in sickle hemoglobinopathies. Am Rev Respir Dis 1993;148(1):249–256.
16. Embury SH, Mohandas N, Paszty C, Crnic L, Cooper P, Cheung ATW. The viscous vicissitudes of in vivo blood flow in the transgenic knockout sickle cell mouse (abstract). Blood 1998;92(10, Suppl 1): 694a.
17. Kasschau MR, Barabino GA, Bridges KR, Golan DE. Adhesion of sickle neutrophils and erythrocytes to fibronectin. Blood 1996;87(2): 771–780.
18. Lowenthal E, Thornley-Brown D, Rutledge L, et al. Correlation of nitric oxide (NO) metabolites with plasma L-arginine in adults with sickle cell disease (SCD). Blood 1996;88(Suppl 1):35 (abstract).
19. Rees DC, Cervi P, Grimwade D, et al. The metabolites of nitric oxide in sickle cell disease. Br J Haematol 1995;91(4):834–837.
20. Lopez BL, Barnett J, Ballas SK, Christopher TA, Davis-Moon L, Ma X. Nitric oxide metabolite levels in acute vaso-occlusive sickle cell crisis. Acad Emerg Med 1996;3(12):1098–1103.
21. Head CA, Brugnara C, Martinez-Ruiz R, et al. Low concentrations of nitric oxide increase oxygen affinity of sickle erythrocytes in vitro and in vivo [see comments]. J Clin Invest 1997;100(5):1193–1198.
22. McDade WA, Shaba HM, Boler N, Mathies S, Nitric oxide increases the solubility of deoxyhemoglobin S solutions and promotes unsickling of sickled cells. Nat Sickle Cell Dis Conf 1997;1:252 (abstract).
23. Enwonwu CO. Increased metabolic demand for arginine in sickle cell anaemia. Med Sci Res 1989;17:997–998.
24. VanderJagt DJ KG, Isichei C, Patuszyn A, Glew RH. Serum and urinary amino acid levels in sickle cell disease. J Trop Pediatr 1997;43(4):220–225.